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Thioredoxin interacting protein suppresses bladder carcinogenesis

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Running head: CXCR4-ERK regulation in bladder cancer by TXNIP

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Abbreviations: BBN, N-butyl-N-(4-hydroxybutyl) nitrosamine; CIS, carcinoma in situ; CXCR4, C-X-C chemokine receptor type 4; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; H&E, hematoxylin–eosin; IGF-1R, type 1 insulin-like growth factor receptor; JNK, c-Jun N-terminal kinase; KO, knock-out; MAPK, mitogen-activated protein kinase; NK, natural killer; pERK, phospho-ERK; SDF-1, stromal cell-derived factor-1; 7TMR, seven-transmembrane receptor; TBP-2, thioredoxin binding protein-2; TUR, transurethral resection; TXNIP, thioredoxin interacting protein; VDUP1, vitamin D3 up-regulated protein 1; WT, wild-type.

Abstract

Thioredoxin interacting protein (TXNIP), which has a tumor-suppressive function, is under-expressed in some human cancers. The function of *TXNIP in vivo* in carcinogenesis is not fully understood. Here, we show *TXNIP* to be down-regulated in human bladder cancer according to grade and stage and also that loss of *TXNIP* expression facilitates bladder carcinogenesis using a mouse bladder cancer model. N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)-induced bladder cancer was found in 100% of *Txnip* knock-out (KO) mice at week 8 of 0.025% BBN administration, but in only 22% of wild-type (WT) mice at the same point. Among growth stimulators, phospho-ERK (pERK) expression was stronger during bladder carcinogenesis in *Txnip*-KO mice than in WT mice. We then evaluated TXNIP's effects on ERK activation through various growth stimulators and their receptors. Over-expression of *TXNIP* in human bladder cancer cells attenuated pERK expression upon stimulation with stromal cell-derived factor-1 (SDF-1), but not with EGF or IGF-1. In *Txnip*-KO mice, immunohistochemical analysis showed enhanced expression of CXCR4, the receptor of SDF-1, and of pERK in urothelial cells during BBN-induced bladder carcinogenesis. Finally, subcutaneous injection of CXCR4 antagonist, TF14016, attenuated pERK in urothelial cells and suppressed bladder carcinogenesis. These data indicate that TXNIP negatively regulates bladder carcinogenesis by attenuating SDF-1-CXCR4-induced ERK activation. This signal transduction pathway can be a potent target in preventing or treating bladder cancer.

Introduction

Bladder cancer is the fifth most common malignancy in the Western society; more than 70,000 new cases of bladder cancer are diagnosed every year in the United States (1). About 70% of patients present with non-muscle invasive bladder tumors, and the remaining 30% present with high-grade invasive tumors at initial diagnosis. Low-grade non-muscle invasive bladder tumors can be treated by transurethral resection with an excellent patient prognosis. But high-grade non-muscle invasive bladder cancer, including carcinoma *in situ* (CIS), tends to invade the muscle layer and metastasize (2), which causes high mortality or severe impairment of quality of life, even if it is cured. The development of bladder tumors is thought to be a multifactorial process and to occur as a direct consequence of the mutagenic actions of chemical or physical carcinogens, such as tobacco smoking. The precise mechanisms underlying carcinogen-induced bladder carcinogenesis have not yet been clarified. To approach this question, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN)-induced mouse bladder carcinoma model has been used widely since the 1970s. One feature of this mouse model is that BBN can induce high-grade non-muscle invasive bladder cancer, followed by muscle invasive bladder cancer, in almost all subject animals (3).

In general, epithelial cells receive oncogenic growth signals that originate in the extracellular environment in response to carcinogenic stimuli (4). Aberrant activation of mitogen-activated protein kinase (MAPK) cascades, such as c-Jun N-terminal kinase (JNK)

and extracellular signal-regulated kinase (ERK), and oncogenic signals involving AKT have been shown to lead to excessive cell growth and division in early-stage carcinogenesis (5). Specifically, up-regulation of oncogenic signaling genes involved in the Ras-Raf-MAPK cascade have been reported in the advanced stages of BBN-induced bladder cancer (6). The MAPKs are activated by receptor signaling pathways, including receptor tyrosine kinases (such as epidermal growth factor receptor [EGFR] and type 1 insulin-like growth factor receptor [IGF-1R]). In addition, seven-transmembrane receptors (7TMRs) have been also reported to activate MAPK signaling. Among the 7TMRs leading to activation of MAPKs, chemokine receptor CXCR4, which is activated exclusively by its ligand stromal cell-derived factor 1 (SDF-1), is up-regulated in many kinds of cancers, including bladder carcinoma. Indeed, CXCR4 was identified as the only chemokine receptor up-regulated in muscle-invasive bladder cancer by screening expression levels of all chemokine receptors in normal urothelium and bladder cancer. (7). It is also up-regulated in high-grade non-muscle invasive bladder cancers including CIS (8), although the role of SDF-1-CXCR4 signaling in bladder carcinogenesis remains unclear.

Thioredoxin interacting protein (TXNIP), also known as thioredoxin binding protein-2 (TBP-2) or vitamin D3 up-regulated protein 1 (VDUP1), is a multi-functional protein. It binds to and inhibits thioredoxin 1 (TRX1), a major redox-regulating molecule, and also plays roles in the development of NK cells, glucose metabolism, cell cycle arrest, and inflammatory

signal regulation (9–11). Regarding the relationship between *TXNIP* and cancer, *TXNIP* expression is down-regulated in gastric, breast, and colorectal cancers (12–15). However, its expression level and role in bladder cancer have not been reported. Moreover, although several growth suppressive mechanisms of TXNIP have been extensively studied *in vitro*, the tumor suppressive functions have not been fully elucidated *in vivo*.

We previously analyzed comprehensive gene expression profiles in recurrent bladder cancer (16,17), and we have found that *TXNIP* is suppressed in bladder cancer. In the present study, we show that *TXNIP* is down-regulated according to grade and stage in human bladder cancers, and that deletion of *Txnip* accelerates BBN-induced bladder carcinogenesis *in vivo*. Additionally, we show that restoration of TXNIP attenuates ERK activation upon SDF-1 stimulation. Furthermore, pharmacological blockade of SDF-1-CXCR4 signaling inhibits *in vivo* BBN-induced bladder carcinogenesis accompanied with marked down-regulation of ERK. Here, we show the significant role of TXNIP on SDF-1-CXCR4-ERK signaling pathway in the molecular mechanisms underlying carcinogen-induced bladder carcinogenesis.

Materials and methods

Quantitative RT-PCR

Total RNA was extracted using an RNeasy Mini kit (QIAGEN, Germany) from cultured cells or whole bladders and reverse-transcribed into cDNA using the First-Strand cDNA Synthesis

Kit (GE Healthcare, Piscataway, NJ). Following primers were used in PCR reactions: human *GAPDH* (forward 5′-GAAGGTGAAGGTCGGAGTC-3′ and reverse 5′-GAAGATGGTGATGGGATTTC-3′), human *TXNIP* (forward 5′-ATGGTGATGTTCAAGAAGATCAAG-3′ and reverse 5′-CTCAGGGGCATACATAAAGA-3′), mouse *Gapdh* (forward 5′-ACCACAGTCCATGCCATCAC-3′ and reverse 5′-TCCACCACCCTGTTGCTGTA-3′), mouse *Txnip* (forward 5′-TCTCCTAGAAGAGCAGCCTACAGG-3′ and reverse 5′-CTCGAAGCCGAACTTGTACTCATA-3′). The real time PCR reaction was performed using the 7300 Real-Time PCR System and RT-PCR reagents (*Power* SYBR Green PCR Master Mix, Applied Biosystems, Tokyo, Japan) according to the manufacturer's protocol. The PCR reactions were done in triplicate; three independent experiments were performed using different sets of samples. Data were normalized relative to the expression level of GAPDH genes. Data in each group were compared with those in another group by post hoc test protected least significant difference (Fisher's PLSD); boxplot graphs were drawn using commercially available software.

Cell culture, stable transfectants, and stimulation by SDF-1α, EGF or IGF-1

Human bladder cancer cell lines: EJ, HT1376, J82, T24, KK47, RT112, RT4, 253J, and TCCSUP were used. The cells were cultured in RPMI 1640 media supplemented with 10%

fetal bovine serum (FBS). Stable 253J and TCCSUP transfectants over-expressing TXNIP were generated as described previously (18) with some modification. The cells were transfected with either pCMV-Tag2b control plasmid or pCMV-Tag2b containing the HA-tagged *TXNIP* gene (HA-TXNIP) using Lipofectamine 2000 (Life Technologies Inc., Grand Island, NY). The expression of Tag2b or HA-TXNIP in the transfectants was confirmed by sequencing, RT–PCR, and western blot analysis. CXCR4 agonist SDF-1α was synthesized by a standard Fmoc-based solid-phase peptide synthesis. Cells were incubated for 48 to 72 h in RPMI media supplemented with 1% FBS and subsequently treated with SDF-1α (100 ng/ml), EGF (50 ng/ml, eBioscience, San Diego, CA), or IGF-1 (250 ng/ml, Cell Sciences, Canton, MA). After 10 or 20 minutes, the cells were collected on ice and analyzed. The timeframe analysis of pERK was performed using SDF-1α (100 ng/ml) in RPMI media without FBS.

Human bladder cancer specimens

Bladder cancer samples (n = 39) were taken by transurethral cold-cup biopsy under direct vision. Non-malignant ureteral urothelia were dissected and isolated by submucosal injection of normal saline from specimens of patients with kidney cancer (n = 6). Patients' characteristics including age, grade, sex, smoking habits, and recurrence-free survival are provided in Table 1. Informed written consent was obtained from each patient before examination.

Txnip-deficient mouse and mouse bladder cancer model

The present animal experiment was approved by the Animal Research Committee of Kyoto University. *Txnip* hetero-knockout (+/–) mice were generated from C57B/6 mice and provided by the Institute for Viral Research, Kyoto University (19). The mice were housed in a specific pathogen-free room. Six- to eight-week-old *Txnip* (–/–) knock-out (KO) mice and wild-type (+/+, WT) mice were given drinking water with or without 0.025% BBN (Tokyo Kasei Kogyo, Tokyo, Japan). In histopathological analysis, bladders were harvested at baseline and after 4, 8, 12, 16, 20, or 24 weeks of treatment (n = 5, 5, 9, 7, 12, 5, or 4 [WT mice]; n = 5, 5, 7, 6, 7, 5, or 3 [*Txnip*-KO mice], respectively). Messenger RNA and total protein was extracted from the excised bladders by mechanically scraping the urothelium and submucosa. For histological examinations, bladders were fixed in 10% buffered formalin. The histopathological diagnosis of the bladder cancer was provided by an experienced pathologist (H. K.) (The 2004 WHO Classification of Bladder Tumors).

Western blot analysis

Total cellular proteins were extracted by disrupting the cells or homogenizing the whole bladders in RIPA buffer. Total cellular protein extracts from cultured cells, mouse bladder tissues, and human bladder urothelium were analyzed by SDS-PAGE. Proteins were transferred to a PVDF membrane, blocked with 1% non-fat dry milk, and incubated with antibodies for the following proteins: HA (Covance Japan, Tokyo, Japan), CXCR4, β-actin

(Abcam, Cambridge, UK), VDUP-1, SDF-1 (Santa Cruz, CA), AKT, phospho-AKT (pAKT, ser473), JNK, phospho-JNK (pJNK, Thr183/Tyr185), ERK, pERK (Cell Signaling Technology Japan, Tokyo, Japan). Stained bands were detected using an ECL-advanced system (GE Healthcare, UK) and a LAS 4000 Mini analyzer and ImageReader software (Fujifilm, Tokyo, Japan). Densitometric scans of the stained bands were performed with ImageJ software (http://rsbweb.nih.gov/ij/). Data were normalized relative to the baseline value in each group. The differences in densitometric values for pERK, pAKT, and pJNK between WT and *Txnip*-KO mice were determined by the Mann-Whitney U test**.**

Immunohistochemistry of mouse bladder cancer

After BBN treatment, mouse bladders were inflated and fixed in 10% formalin. Mice without BBN treatment were used as negative controls. Human bladder cancer specimens obtained by transurethral cold-cup biopsy were fixed in 10% formalin overnight. The tissues were embedded in paraffin and were stained with hematoxylin and eosin in accordance with standard procedures. The formalin-fixed and paraffin-embedded bladders were stained as described previously (8) with antibodies for VDUP1 (1:200 dilution) or pERK (1:100 dilution). The specificity of the staining was confirmed using the corresponding rabbit or mouse IgG as negative controls for primary antibodies. Each tissue sample was observed by an eclipse E1000 microscope (Nikon, Tokyo, Japan).

Treatment with CXCR4 antagonist TF14016

A potent CXCR4 antagonist, 4F-benzoyl-TN14003 (TF14016), was synthesized as described previously (20). TF14016 was dissolved with sterile water. TF14016 (7.5 mg/kg) was injected subcutaneously into nine each wild-type and *Txnip*-KO mice, every other day, in conjunction with 0.025% BBN administration. After 12 weeks of BBN administration, bladders were harvested 30 minutes after injection of TF14016 and analyzed using immunohistochemistry or western blot analysis.

Results

Txnip expression is down-regulated in human bladder cancers and in the BBN-induced bladder cancer model.

Expression of *TXNIP* mRNA in human bladder cancers and bladder cancer cell lines was analyzed by quantitative RT-PCR. As shown in Figure 1A, *TXNIP* mRNA expression was not evident in all seven bladder cancer cell lines examined, and significantly decreased in human bladder cancers (n = 39) compared to normal urothelium (n = 6) ($P < 0.0001$). Its expression correspondingly decreased according to the grade and stage of the cancer ($P = 0.026$).

Next, the expression levels of *Txnip* mRNA were analyzed in BBN-induced mouse bladder cancers. Under our experimental condition, CIS started to develop after 8 weeks of BBN administration, and lesions were identified in 86% (6 out of 7) of the mice after 12

weeks of BBN administration. Sub-mucosal invasion (pathological T1 or more) was identified in all mice (n = 5) at 20 weeks of BBN administration (Figure 1B).Over the course of the BBN-induced bladder carcinogenesis, *Txnip* expression declined time-dependently and was reduced to about 24%, in average, of the baseline level at 20 weeks of BBN administration (Figure 1C). Interestingly, a 37% decrease in *Txnip* expression was observed at 4 weeks of BBN administration, at which no malignant changes were identified histologically. These data indicated that the expression of TXNIP gene was suppressed in the course of human and mouse bladder carcinogenesis.

Knock-out of Txnip accelerates BBN-induced bladder carcinogenesis

To determine whether the decreased expression of *Txnip* contributes to the development of bladder cancer, the prevalence of bladder cancer in *Txnip*-KO mice was compared with that in WT mice, using the BBN-induced bladder carcinogenesis model. Bladder cancer was pathologically identified in 60% (3 out of 5) and 100% (7 out of 7) of the *Txnip*-KO mice at 4 and 8 weeks of BBN administration, respectively, whereas it was identified in none of 5 and only 22% (2 out of 9) of the WT mice at 4 and 8 weeks, respectively (Figures 2A and B). When focusing on high-grade CIS, *Txnip*-KO mice developed this type of bladder cancers about 4 weeks earlier than WT mice (Figure 2C). Moreover, invasive bladder cancer (pT1 or more) and/or squamous cell carcinoma (SCC), which have characteristic histological features to advanced BBN-induced mouse bladder cancers, were observed also about 4 weeks earlier

in the *Txnip*-KO mice than in the WT mice (Figure 2D). Thus, knocking-out *Txnip* accelerated BBN-induced bladder carcinogenesis, suggesting that TXNIP plays a suppressive role for bladder carcinogenesis. Although bladder carcinogenesis in WT mice is delayed, it is not prevented and is similar to the carcinogenesis in WT mice at 16-20 weeks of BBN treatment. This is likely because by that time TXNIP levels were similar in the two groups as shown in Figure 2E. TXNIP level in bladder urothelium was down-regulated at 12 and 20 weeks of BBN treatment compared to baseline in WT mice (n=3 in each group, Figures 2E).

Phosphorylation of ERK is enhanced in Txnip-KO mouse bladders during BBN-induced bladder carcinogenesis

To investigate the molecular mechanisms underlying bladder carcinogenesis in *Txnip*-KO mice, the activation levels of oncogenic growth-stimulatory signals were analyzed during BBN-induced bladder carcinogenesis in the WT and *Txnip*-KO mice. The phosphorylation levels of AKT, JNK, and ERK were up-regulated at 8 weeks compared to baseline (0 week) in both WT and *Txnip*-KO mice (n = 3 in each group, Figure 3A). Among them, upregulation of pERK was about 4-fold enhanced in *Txnip*-KO mice compared to WT mice at 8 weeks of bladder carcinogenesis ($P < 0.05$), whereas there were no obvious differences in the up-regulation of pAKT and pJNK between *Txnip*-KO and WT mice. Immunohistochemical analysis demonstrated that the staining for pERK was enhanced in urothelial cells after 8 weeks of BBN administration in *Txnip*-KO mice (Figure 3B). Thus, loss of *Txnip* resulted in

enhanced activation of ERK in bladder urothelium as well as accelerated BBN-induced bladder carcinogenesis.

Overexpression of TXNIP attenuated SDF-1/CXCR4 induced phosphorylation of ERK in human bladder cancer cells

To investigate the impact of TXNIP on ERK activation induced by various growth stimulations, such as EGF, IGF-1 or SDF-1, three stable transfectants with HA-tagged *TXNIP* were established from 253J and TCCSUP bladder cancer cell lines (253J-HA-TXNIP-1, 2, and TCCSUP-HA-TXNIP). Western blot analysis revealed that phosphorylation levels of ERK upon SDF-1 stimulation was lower in 253J-HA-TXNIP-1, -2, and TCCSUP-HA-TXNIP than in control cell lines (Figures 4A, B, and S1). On the other hand, there were no differences in pERK upon EGF or IGF-1 stimulation between 253J-HA-TXNIP-2 and control cells (Figure 4C). Similar results were observed in the other two stable transfectants (253J-HA-TXNIP-1 and TCCSUP-HA-TXNIP) upon EGF or IGF-1 stimulation (data not shown). There were also no differences in the expression levels of corresponding receptors, including EGFR, IGFR, CXCR4, phospho-EGFR, or phospho-IGF-1R (data not shown). These results indicate that overexpression of TXNIP attenuates SDF-1-CXCR4-induced activation of ERK exclusively.

CXCR4 antagonist TF14016 suppresses pERK and BBN-induced carcinogenesis in Txnip-KO mice

The expression status of SDF-1, CXCR4, ERK and pERK were assessed in BBN-induced bladder carcinogenesis. SDF-1 and ERK were constantly expressed until 8 weeks of BBN administration, whereas CXCR4 was gradually up-regulated in the course of bladder carcinogenesis in the WT mice. When *Txnip*-KO mice were compared with WT mice, there were no obvious differences in the expression of SDF-1 and CXCR4. On the other hand, the expression of pERK was more enhanced in *Txnip*-KO mice than in the WT mice (Figure 5A).

To assess the impact of activation of ERK induced by SDF-1-CXCR4 stimulation on enhancement of bladder carcinogenesis in *Txnip*-KO mice, SDF-1-CXCR4 signaling was blocked by a specific CXCR4 antagonist TF14016 in both WT mice and *Txnip*-KO mice. TF14016 was injected subcutaneously into WT mice and *Txnip*-KO mice every other day for 12 weeks during BBN administration. No significant adverse effects, including weight loss, were observed due to TF14016. Western blot analysis demonstrated that TF14016 markedly attenuated pERK in the mouse bladder of both groups to the similar level after 12 weeks of BBN consumption (Figure 5B). Pathological examination revealed that the prevalence of malignant bladder lesion was remarkably lower in TF14016-treated WT mice and *Txnip*-KO mice than in each control mice (Figures 5C and D).

Discussion

The present study shows novel findings that expression of *Txnip* mRNA is suppressed in human bladder cancers and in a mouse model of BBN-induced bladder carcinogenesis. Moreover, we have clearly demonstrated that loss of *Txnip* facilitates BBN-induced mouse bladder carcinogenesis using *Txnip*-KO mice (Figure 6). In general, a tumor suppressor gene is defined as a gene whose partial or complete inactivation leads to an increased likelihood of cancer development (4). *Txnip* is thought to be a tumor suppressor gene based on observational evidence of suppressed expression in cancer tissues from various origins (12–15) and functional verification using cultured cancer cells in *in vitro* experimental series (9–11). Reportedly, *Txnip*-deficient mice are also predisposed to renal (21) and hepatocellular (22,23) carcinomas. Our data, which indicates that TXNIP functions as a tumor suppressor in urothelium, is consistent with its decreased expression in several kinds of cancers.

We have investigated *in vivo* functional consequences of TXNIP loss using a BBN-induced bladder carcinogenesis model in the *Txnip*-KO mice. In general, carcinogenic stimuli, such as BBN, boost oncogenic growth-stimulatory signals, leading to excessive cell growth and division at the early stage of carcinogenesis (5). Therefore, we investigated ERK, JNK and AKT signaling as major growth-stimulatory signals, and found that ERK was more active in *Txnip*-KO mice than in WT mice. Aberrant activation of ERK, a second messenger involved in an important oncogenic signal transduction pathway, is reportedly associated with

bladder carcinogenesis, among other cancers. On the other hand, some possible mechanisms of TXNIP have been listed in tumor suppression. A previous *in vitro* study reported that TXNIP decreases cell proliferation by restricting cell cycle progression; TXNIP deficiency reduced expression of p27, a cyclin-dependent kinase inhibitor (24). Alteration of redox status has been also proposed as a mechanism of tumor suppression, since TXNIP inhibit the activity of TRX1. However, under the conditions we used to induce bladder carcinogenesis, we could not find obvious differences in the expression of TRX1, cell-cycle related genes including p27, and the status of redox (8-hydroxy-2′-deoxyguanosine [8-OHdG]) between *Txnip*-KO mice and WT mice (data not shown). Collectively, TXNIP can function as a tumor suppressor mainly through regulating ERK activity, which is strongly associated with oncogenic process toward bladder carcinogenesis. Since the present study remains observational, the precise mechanism of action should be clarified in the next studies.

Phosphorylation of ERK can occur via activation of either of several signal pathways including receptor tyrosine kinases (i.e., IGF-1R and EGFR) or 7TMRs (i.e., CXCR4). In the present study, BBN administration induced both CXCR4 expression in urothelial cells and SDF-1 expression in stromal mesenchyme cells. In contrast, bladder cancer cell lines transfected with *TXNIP* demonstrated attenuated CXCR4-ERK activation upon SDF-1 stimuli. Therefore, CXCR4 is considered to be a major up-stream receptor activating ERK signaling; it plays an important role in bladder carcinogenesis by stimulating several transcription

factors. Indeed, CXCR4 antagonist significantly attenuates ERK activation and suppresses bladder carcinogenesis in both WT and *Txnip*-KO mouse model, to almost the same level. Since the effects of TXNIP deletion is abrogated by CXCR4 antagonist, there seems to be a causal link between TXNIP deletion and enhanced bladder carcinogenesis. These results suggest that TXNIP suppression facilitates bladder carcinogenesis by enhancing CXCR4–ERK activation.

Another important upstream regulator of ERK is the Ras signaling pathway, which is activated by receptor tyrosine kinases such as IGF-1R, EGFR and FGFR3. Aberrant activation of Ras oncoproteins is associated with development of low-grade non-muscle invasive bladder cancer (25). However, BBN-induced mouse bladder cancer shows a morphologic effect similar to another bladder carcinogenesis pathway, namely high-grade CIS muscle-invasive cancer pathway (25). In the present study, restoration of *TXNIP* did not alter ERK activation upon IGF-1 or EGF stimuli; and CXCR4 blockade suppressed BBN-induced carcinogenesis. Also, TXNIP expression is significantly more suppressed in high-grade muscle-invasive human bladder cancers than in low-grade non-muscle-invasive types of cancer. These findings suggest that activation of SDF-1-CXCR4 signaling drives a CIS muscle-invasive bladder cancer pathway, and that a major effect of TXNIP loss is to facilitate this pathway. Recent reports support the notion that the ERK pathway plays an important role in muscle invasive bladder cancer (26–29). Because TXNIP expression is also suppressed in

low-grade non-muscle-invasive bladder cancers compared to normal urothelia, loss of *TXNIP* may play other roles in bladder carcinogenesis, which should be elucidated in future studies.

Using a BBN-induced bladder carcinogenesis mouse model, we have shown that CXCR4 antagonist TF14016 can reduce bladder carcinogenesis. The 14-mer peptide T140 was developed from structure-activity relationship studies of two antibacterial and antiviral peptides; 17-mer peptide tachyplesins and 18-mer peptide polyphemusins isolated from the Japanese horseshoe crab (*Tachypleus tridentatus*) and the American horseshoe crab (*Limulus polyphemus*), respectively. T140 possesses potent anti-HIV activity by binding specifically to CXCR4 and TF14016 is a potent and bio-stable T140 analog (20). To our knowledge, this is the first report of successful bladder cancer prevention using a drug that attenuates a specific oncogenic signal. A limitation of the present study is that the therapeutic effect of TF14016 on established bladder tumors have not been clarified. Therefore, it seems the most reasonable and practical to use this drug to prevent recurrence of high-grade bladder cancer after transurethral resection (TUR). Prevention of post-TUR bladder cancer recurrence is a major clinical challenge, although some anti-cancer drugs and Bacillus Calmette-Guérin (BCG) are currently available with partial effects. Although some newly developed antigen-based drugs and molecular-targeted agents have been tested for this purpose (30), none of these novel treatments has been established so far. Peptide-based TF14016 and its analogs also offer advantages in terms of cost over other novel treatment modalities. Although we did not find

any lethal reaction or significant weight loss during the 12 weeks' treatment, further evaluation of acute and chronic adverse events is essential for the future clinical use.

In conclusion, TXNIP, which is silenced during bladder carcinogenesis, suppresses ERK activation in the SDF-1-CXCR4 signaling and also bladder carcinogenesis, suggesting that SDF-1-CXCR4-ERK signaling might be a good target to prevent bladder cancer.

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Figure legends

Fig. 1. Messenger RNA level of *TXNIP* gene is suppressed in bladder cancers. (**A**) Compared with normal urothelium, *TXNIP* is suppressed in human bladder cancers. Particularly in high grade and/or high stage (pT1 or more) cancers *TXNIP* is strongly suppressed. All bladder cancer cell lines express little if any *TXNIP*. (**B, C**) *Txnip* is suppressed in a BBN-induced bladder cancer model. Bladder carcinoma *in situ* (CIS) developed after 8–12 weeks of BBN administration in WT mice (**B**). (**C**) Sequential change of mRNA of *Txnip* in BBN-induced mouse bladder carcinogenesis. *Txnip* is suppressed at 4 weeks of BBN administration and declined time-dependently over the course of BBN administration; n = 3 in each group. Statistical analysis: Post-hoc test (Fisher's PLSD). (**D**) Western blot for TXNIP in BBN-induced mouse bladder carcinogenesis. TXNIP is down-regulated at 8 weeks of BBN consumption and down-regulated over the course of BBN administration.

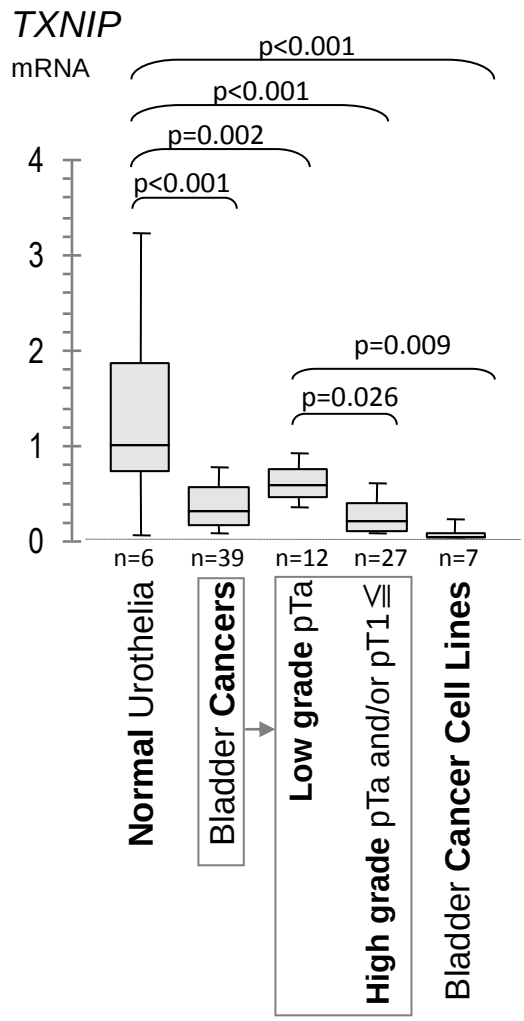
Fig. 2. *Txnip*-KO mice develop BBN-induced bladder cancer earlier than WT mice. (**A**) Representative histologies of mouse bladder in WT mice (top) and *Txnip*-KO mice (bottom). H&E staining, original magnification × 200 (NCT) and × 100 (8 and 12 weeks). (**B–D**) Histograms showing ratio of bladder cancer according to duration of BBN treatment. Overlaid line indicates mean Txnip levels of WT mice at each time point relative to the baseline. (**B**) Overall cancers; (**C**) high grade cancer; (**D**) invasive cancer (pT1 or more) and/or SCC. (**E**) Representative immunohistochemistry for TXNIP in WT mouse bladder

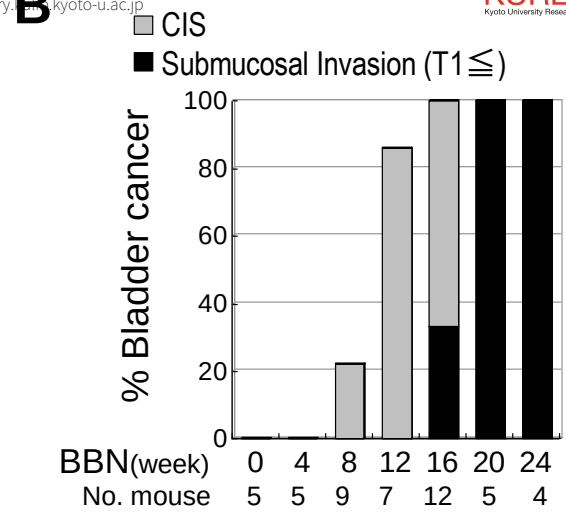
cancer. TXNIP level in bladder urothelia was down-regulated in CIS at 12 weeks and T1 cancer at 20 weeks of BBN treatment compared to baseline (0 week) in WT mice (Figures 2D).

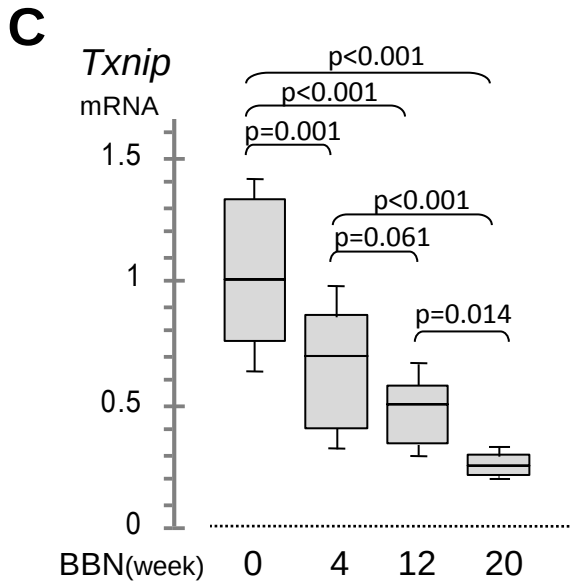
Fig. 3. (**A**) Western blot for ERK, AKT, and JNK signaling. Phosphorylation levels of ERK, AKT, and JNK are up-regulated at 8 weeks of BBN consumption compared to baseline. pERK is more up-regulated in *Txnip*-KO mice than in WT mice at 8 weeks of BBN consumption. The chart below shows densitometric values for pERK, pAKT, and pJNK relative to the baseline. (**B**) Representative immunohistochemistry for pERK in *Txnip*-KO mouse bladder cancer. pERK are up-regulated in urothelial cells after 8 weeks of BBN administration. Original magnification × 200.

Fig. 4. (**A**) Forced expression of TXNIP attenuates pERK response to SDF-1 in human bladder cancer cell lines. The cell line 253J-HA-TXNIP-1 and 2 showed decreased activation of pERK after SDF-1 treatment compared with the control cells (arrowhead). Similar results were obtained from TCCSUP-HA-TXNIP. Densitometric values are shown in the chart below. (**B**) Timeframe analysis of pERK activation after SDF-1 treatment in FBS-free medium. Lower panel shows time-dependent changes in densitometric values for pERK. Similar results were obtained from TCCSUP-HA-TXNIP. (**C**) Forced expression of TXNIP did not influence major receptor tyrosine kinase pathways including EGFR and IGF-1R signaling.

Fig. 5. (**A**) Western blot for SDF-1, CXCR4 and ERK in BBN-induced bladder carcinogenesis of both WT and *Txnip*-KO mice. CXCR4 is induced at 2 weeks of BBN administration in both WT and *Txnip*-KO mice, whereas the SDF-1 level did not show obvious changes. On the other hand, pERK was more enhanced in *Txnip*-KO mice than in the WT mice. (**B**–**D**) A specific CXCR4 antagonist TF14016 suppressed BBN-induced ERK activation and bladder carcinogenesis in *Txnip*-KO mice. (**B**) TF14016 suppressed pERK after 12 weeks of BBN administration in both WT mice and *Txnip*-KO mice. (**C**) Representative histology of mouse bladder. TF14016 suppressed bladder carcinogenesis after 12 weeks of BBN administration in both WT mice and *Txnip*-KO mice. Original magnification × 200. (**D**) TF14016 reduced the incidence of bladder carcinogenesis after 12 weeks of BBN administration in *Txnip*-KO mice to almost the same level as observed in WT mice.







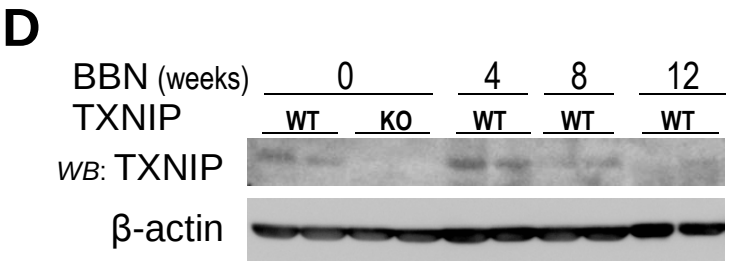
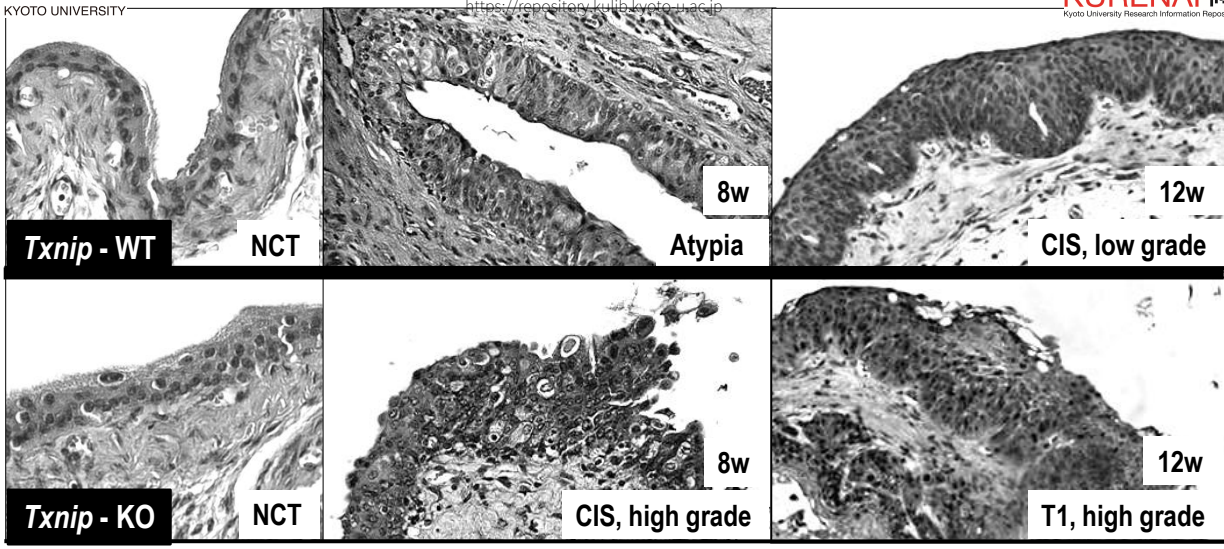
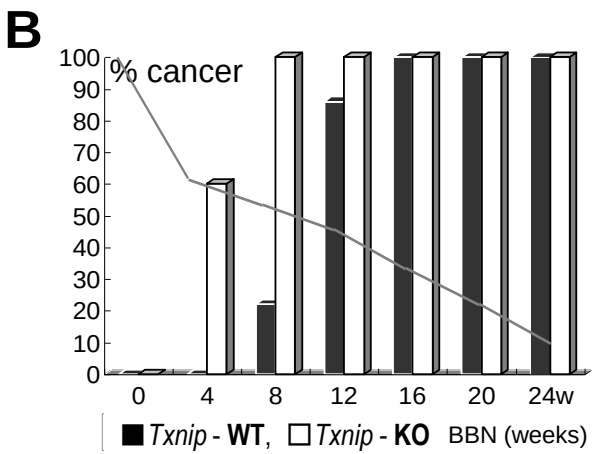
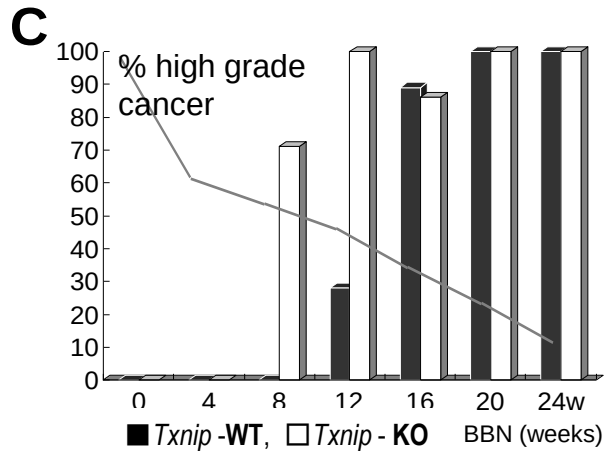


Fig. 1







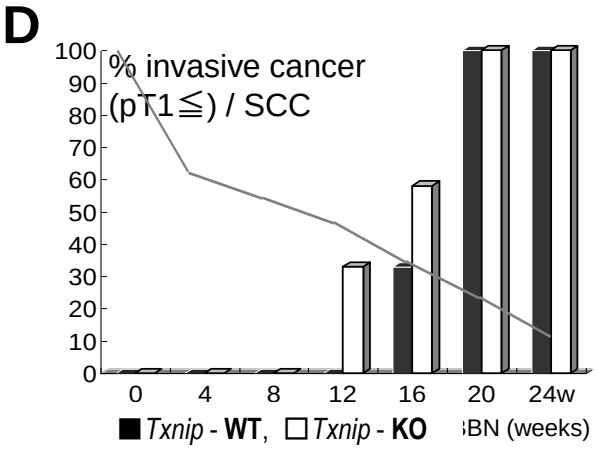
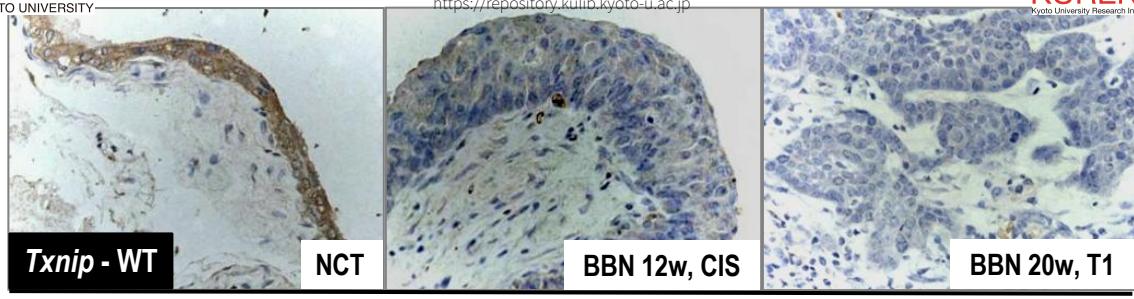
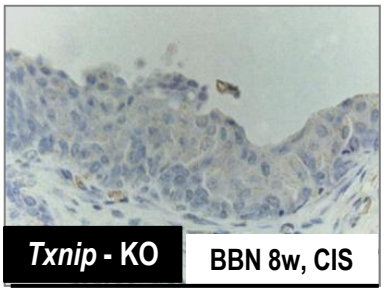


Fig. 2





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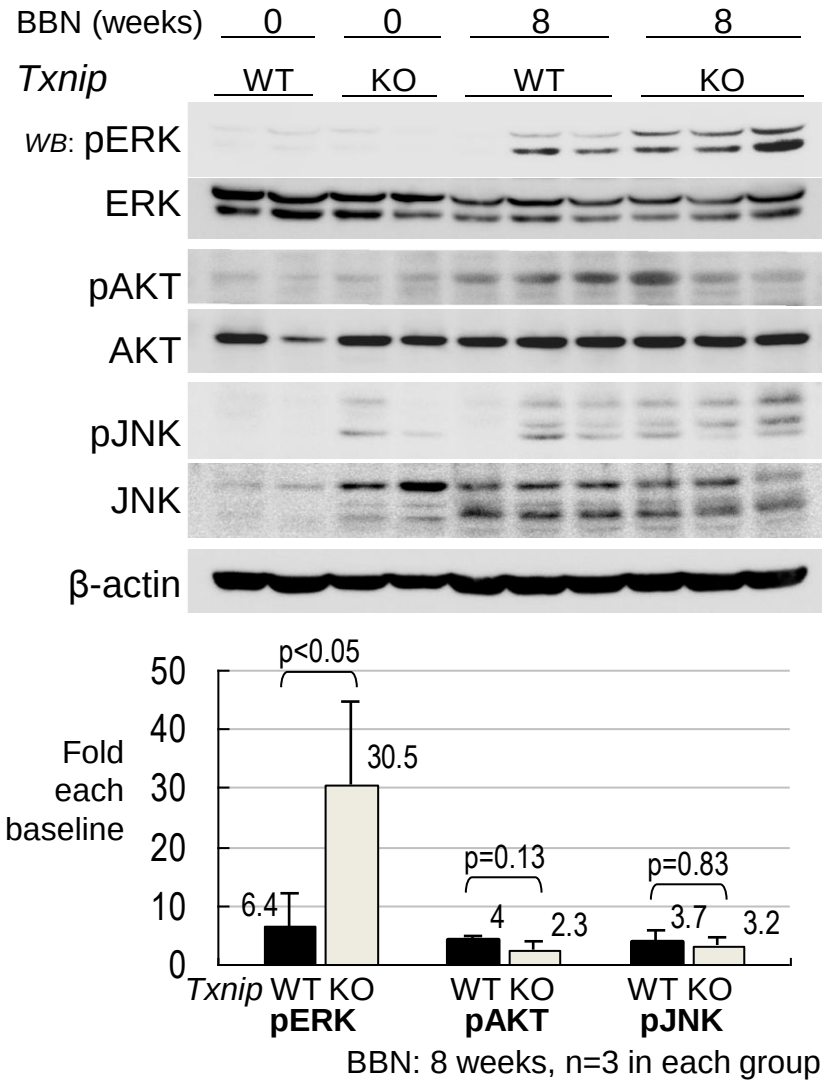

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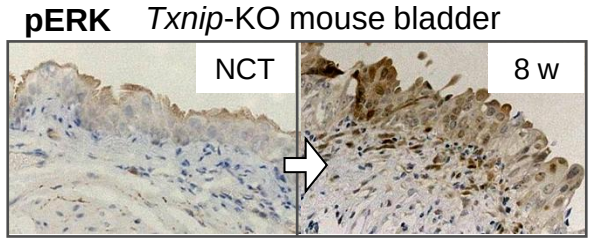

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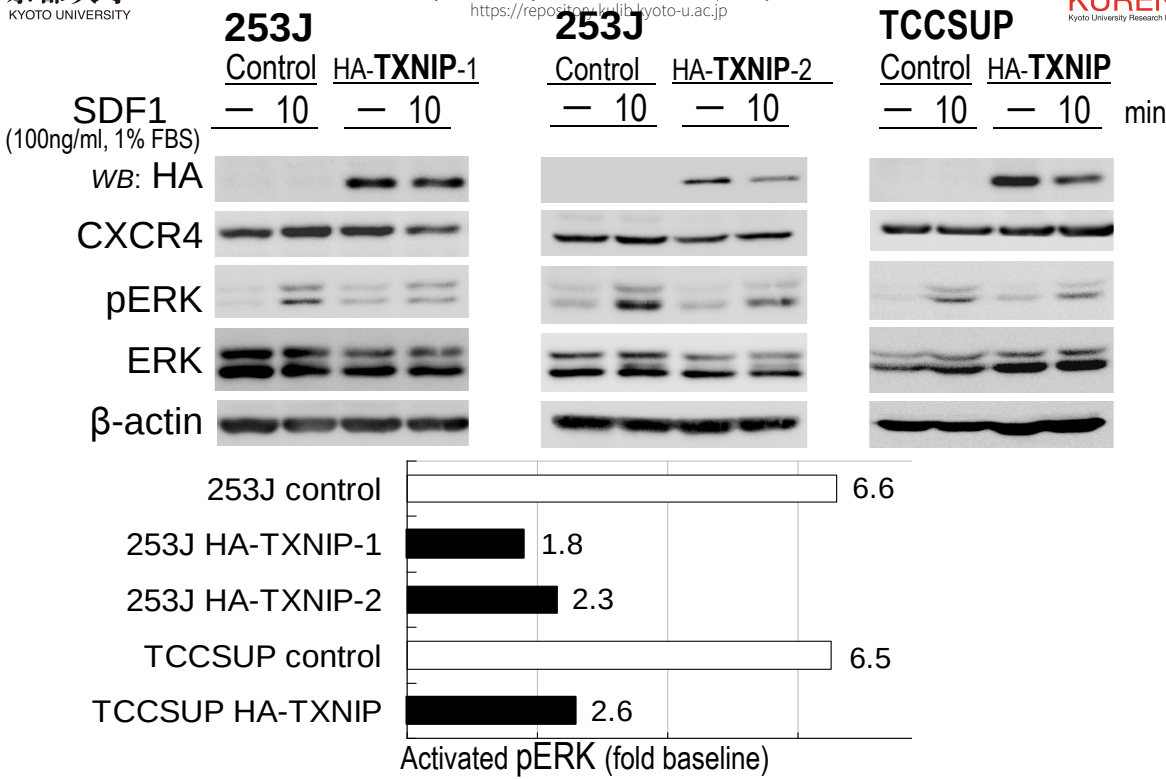

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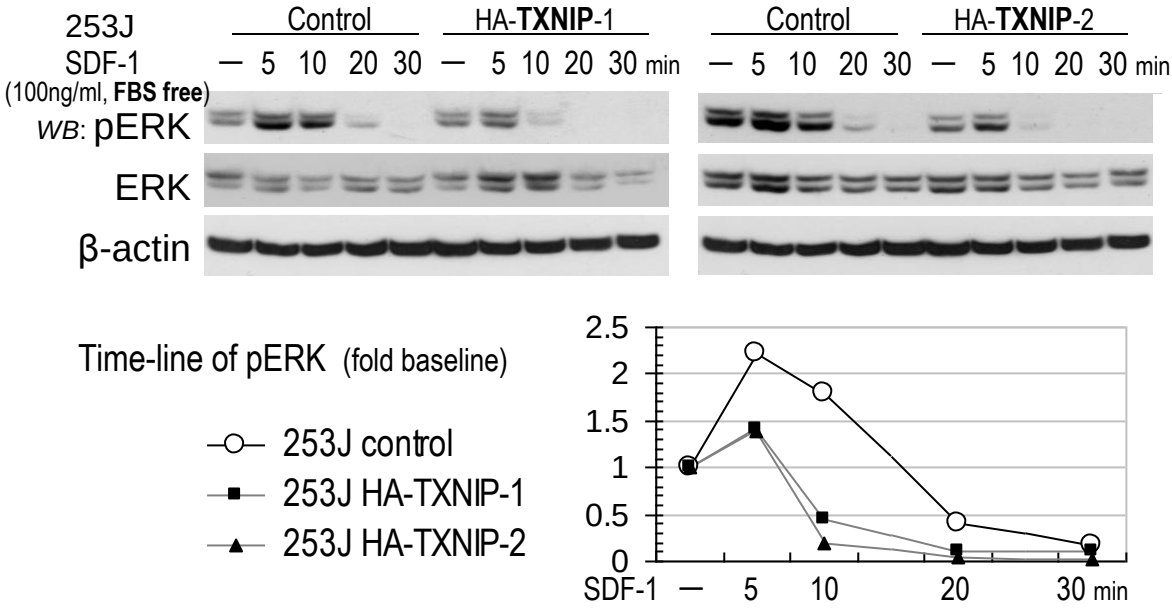
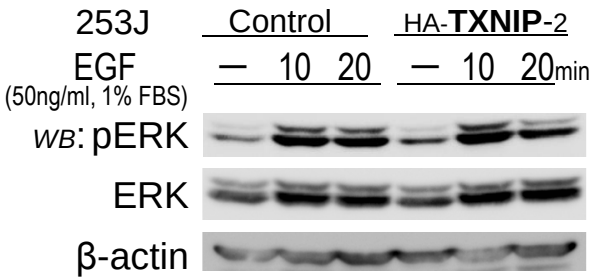
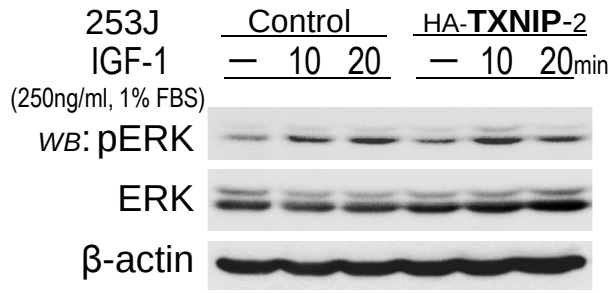
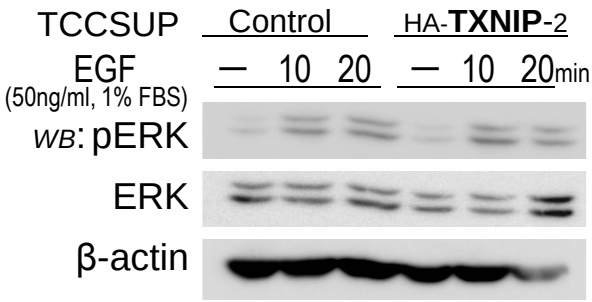
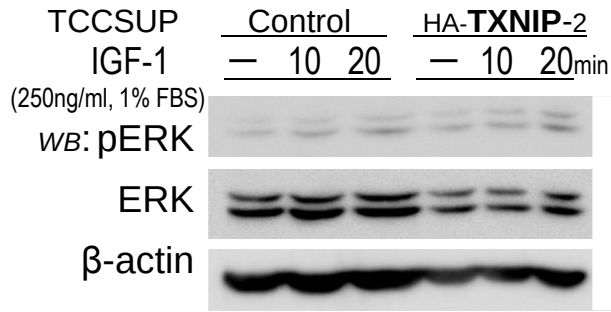


Fig. 4









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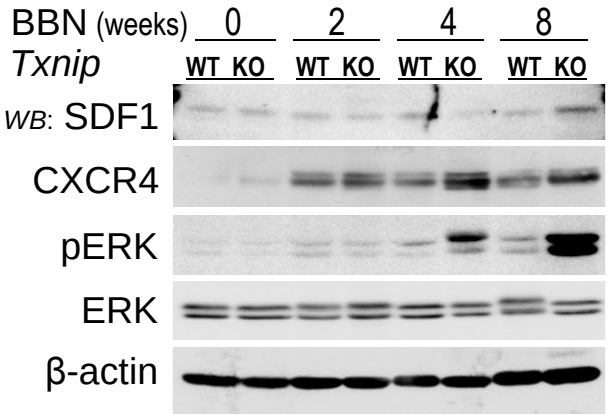

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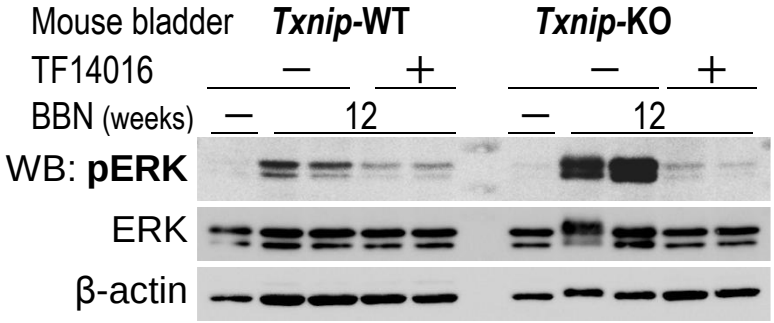
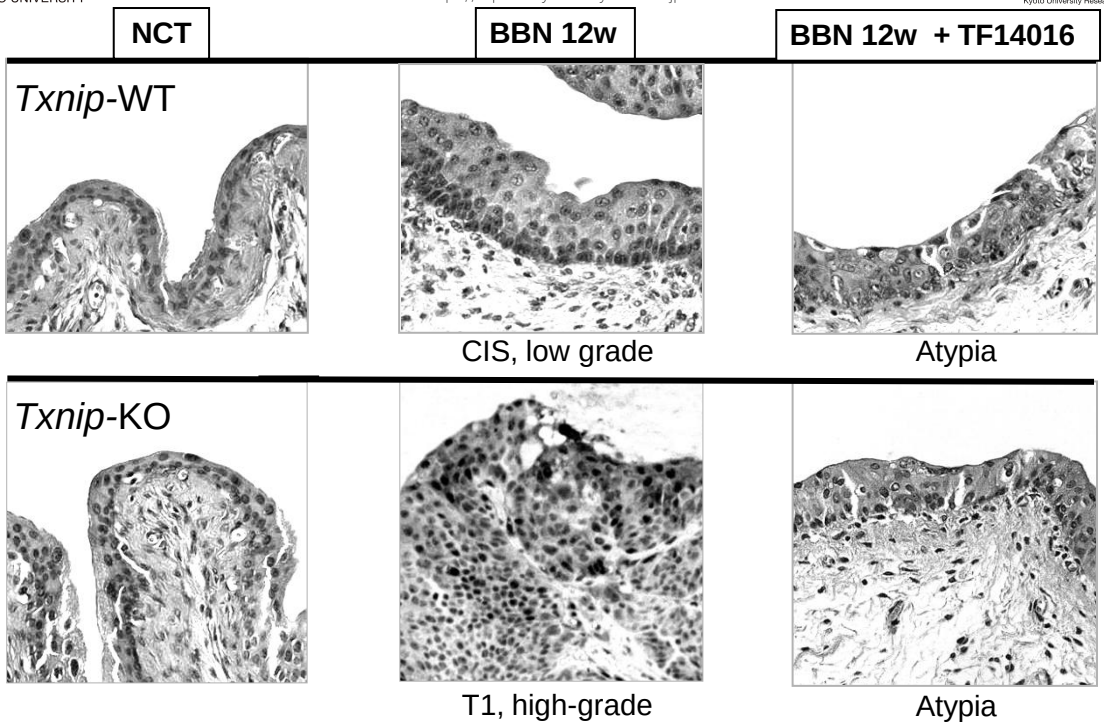


Fig. 5



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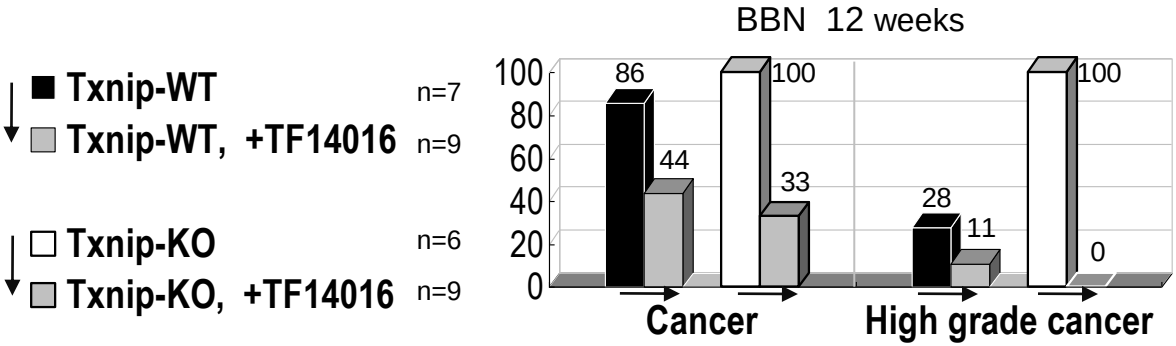


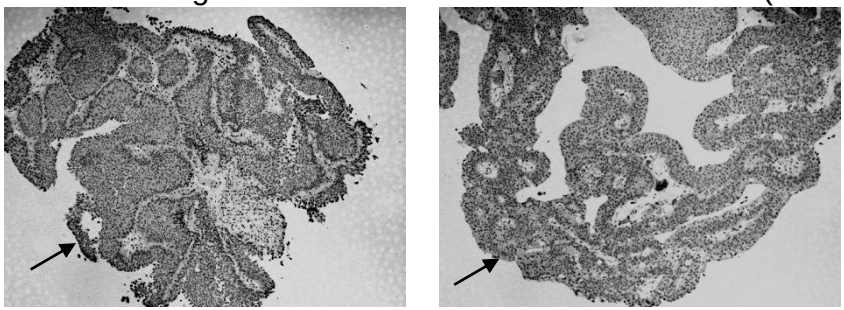
Fig. 5

Table 1. Patients' characteristics of 39 bladder cancers and 6 normal urothelial samples including age, grade, sex, smoking habits, 5 year recurrence-free survival. Briskman's index: daily tobacco smoke times years. *: cystectomy is performed followed by transurethral resection.

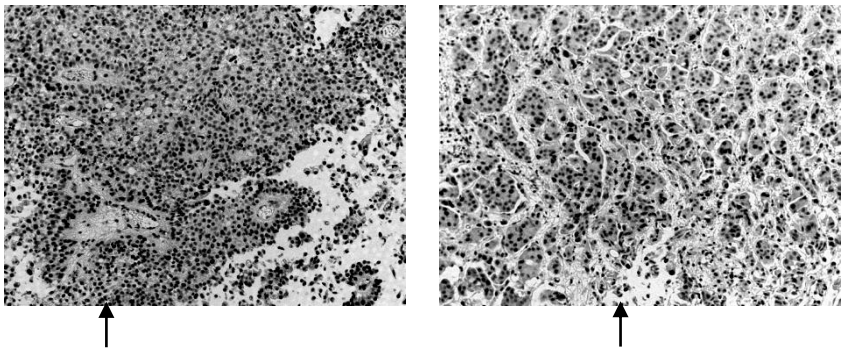
Group	Normal urothelium	Bladder caner		
		Low grade and low stage	High grade and/ or high stage	Total
Patients (No.)	6	12	27	39
Age [average (S.D.)]	67.5 (7.0)	66.3 (12.0)	72.7 (14.7)	70.7 (14.1)
Sex (% male)	83	75	72	73
Stage (%Ta	-	100	0	32
T1	-	0	20	7
T2	-	0	32	22
T3	-	0	40	27
T4)	-	0	16	11
% Tobacco smoker	33	25	32	30
Briskman's index [average (S.D.)]		108 (208)	309 (807)	247 (683)
% 5yr recurrence-free survival	-	50	41*	44

Table 1

Human low grade and non-muscle invasive cancers (arrows).

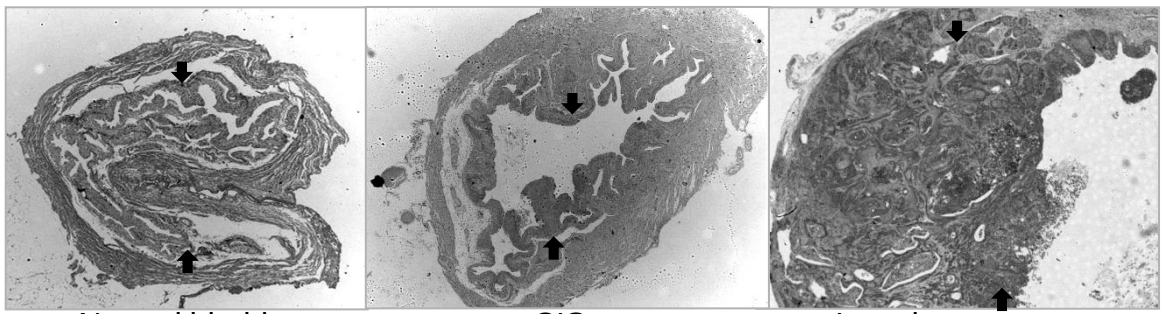


Human high grade and muscle-invasive bladder cancers (arrows).



B

Mouse bladders. Arrows: urothelium or cancer.



Normal bladder CIS Invasive cancer

Fig. S1

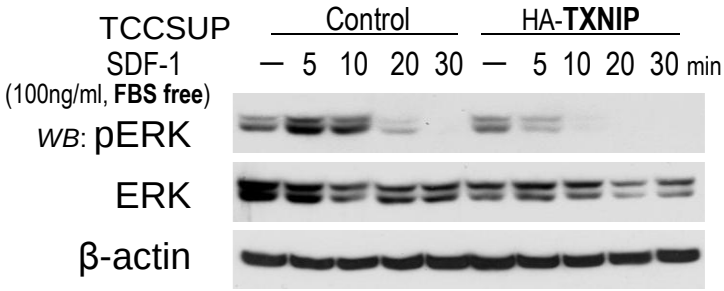


Fig. S2

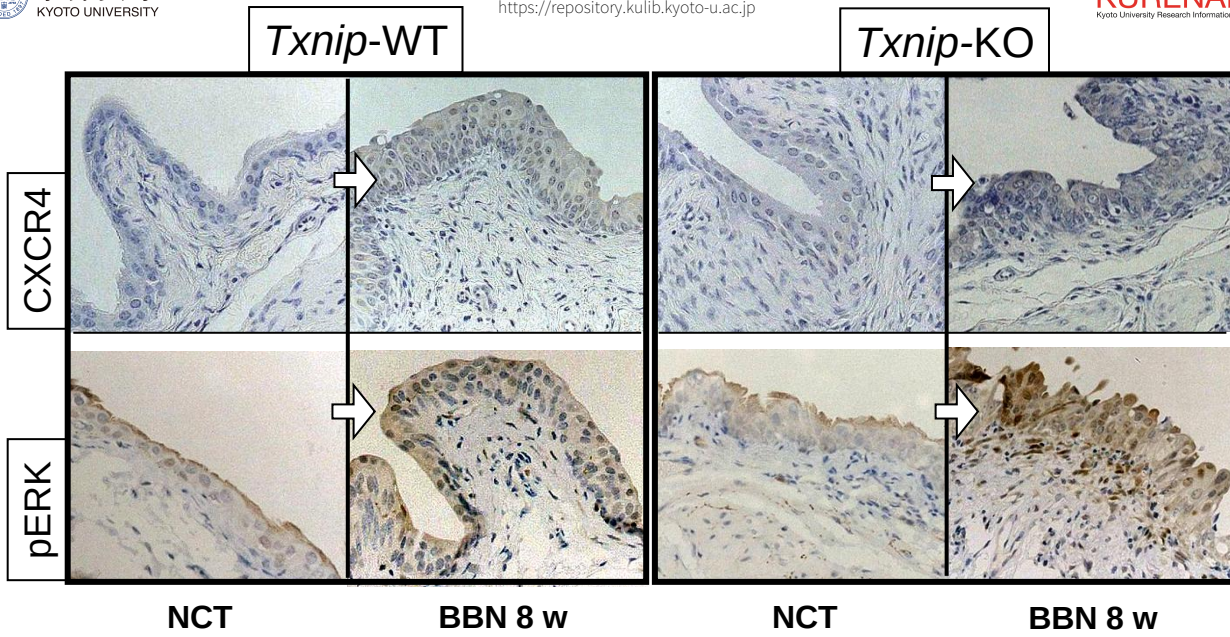


Fig. S3